Title
Distinct functions of integrin alpha and beta subunit cytoplasmic domains in cell spreading and formation of focal adhesions.

Journal
The Journal of cell biology, 122(1)

ISSN
0021-9525

Authors
Ylänne, J
Chen, Y
O'Toole, TE
et al.

Publication Date
1993-07-01

DOI
10.1083/jcb.122.1.223

Peer reviewed

Distinct Functions of Integrin α and β Subunit Cytoplasmic Domains in Cell Spreading and Formation of Focal Adhesions

J. Ylänne, Y. Chen, T. E. O'Toole, J. C. Loftus, Y. Takada, and M. H. Ginsberg

Committee on Vascular Biology, The Scripps Research Institute, La Jolla, California 92037

Abstract. Integrin-mediated cell adhesion often results in cell spreading and the formation of focal adhesions. We exploited the capacity of recombinant human $\alpha_{IIb}\beta_3$ integrin to endow heterologous cells with the ability to adhere and spread on fibrinogen to study the role of integrin cytoplasmic domains in initiation of cell spreading and focal adhesions. The same constructs were also used to analyze the role of the cytoplasmic domains in maintenance of the fidelity of the integrin repertoire at focal adhesions. Truncation mutants of the cytoplasmic domain of α_{IIb} did not interfere with the ability of $\alpha_{IIb}\beta_3$ to initiate cell spreading and form focal adhesions. Nevertheless, deletion of the α_{IIb} cytoplasmic domain allowed indiscriminate recruitment of $\alpha_{IIb}\beta_3$ to focal adhesions formed by other integrins. Truncation of the β_3 subunit cytoplasmic domain abolished cell spreading mediated by $\alpha_{IIb}\beta_3$ and also abrogated recruitment of $\alpha_{IIb}\beta_3$ to focal adhesions. This truncation also dramatically impaired the ability of $\alpha_{IIb}\beta_3$ to mediate the contraction of fibrin gels. In contrast, the β_3 subunit cytoplasmic truncation did not reduce the fibrinogen binding affinity of $\alpha_{IIb}\beta_3$. Thus, the integrin β_3 subunit cytoplasmic domain is necessary and sufficient for initiation of cell spreading and focal adhesion formation. Further, the β_3 cytoplasmic domain is required for the transmission of intracellular contractile forces to fibrin gels. The α subunit cytoplasmic domain maintains the fidelity of recruitment of the integrins to focal adhesions and thus regulates their repertoire of integrins.

Cell adhesion is controlled by binding affinity and kinetics of interaction between adhesive ligands and cell surface receptors. In addition, events such as lateral diffusion of receptors (7) and interactions with and reorganization of the cytoskeleton strengthen adhesion (16, 29). Cell adhesion induces changes of cell shape and cytoskeletal organization and regulates cell growth and patterns of gene expression (24, 48, 53). Integrins are transmembrane heterodimeric glycoprotein adhesion receptors present in almost all cells (23). These receptors mediate all of the consequences of cell adhesion enumerated above. Moreover, after initial cell adhesion, many integrins are concentrated at sites of close approximation between cell and substrate. These sites, termed focal adhesions or focal contacts (4), are also sites of end-on insertion of actin microfilaments into the plasma membrane and at which certain cytoskeletal and signaling molecules concentrate (53). Ligand binding to many integrins triggers focal adhesion formation. In addition to initiating focal adhesions, certain mutant integrins (58) or receptor chimeras containing the β_1 cytoplasmic domain (27, 49) are recruited to existing focal adhesions without evident ligand binding. Thus, analysis of the role of integrins in formation of focal adhesions may be divided into initiation and recruitment functions.

Integrin cytoplasmic domains are topographically accessible to intracellular cytoplasmic components. Moreover, the β_1 cytoplasmic domain binds to focal adhesion proteins such as talin (22) and α-actinin (41). The β_1 cytoplasmic domain is required for localization of recombinant $\alpha_5\beta_1$ to existing focal adhesions (19, 31, 56), but the presence of endogenous β_1 precluded analysis of initiation in the published work. Preliminary studies reported that the α_5 subunit cytoplasmic domain is not absolutely required for focal adhesion formation (Juliano, R. L., J. S. Bauer, L. J. Kornberg, and J. Varner. 1992. *Mol. Biol. Cell.* 3[Suppl]:94a).

Integrin $\alpha_{IIb}\beta_3$ (platelet GPIIb–IIIa) is a prototype integrin that mediates cell–cell and cell-substratum interaction (43, 44), signals cell spreading (61) and initiates and enters focal adhesions (64, 65). Moreover this integrin is required for the retraction of fibrin clots (5, 37), wherein intracellular contractile forces are transmitted to extracellular fibrin polymers. $\alpha_{IIb}\beta_3$ offers several advantages in the studies of integrin domains which function in cell adhesion. (*a*) Certain ligands such as fibrinogen (fg)[1] and von Willebrand factor promote adhesion via $\alpha_{IIb}\beta_3$, but not through the majority of other integrins. Thus, in many cell lines, events mediated by transfected $\alpha_{IIb}\beta_3$, such as cell adhesion, spreading, and focal adhesion formation, can be analyzed with minimal confounding effects of endogenous integrins. (*b*) Well character-

J. Ylänne's present address is Department of Biochemistry, University of Helsinki, Helsinki, Finland.

This is publication #7148-CVB from the Scripps Research Institute.

1. *Abbreviation used in this paper*: fg, fibrinogen.

ized quantitative ligand binding assays for $\alpha_{IIb}\beta_3$ are available (32), again without significant contributions from endogenous integrins. (*c*) An extensive library of $\alpha_{IIb}\beta_3$ mAbs exists. Antibodies specific for occupied (13,14) or activated (54) conformations of $\alpha_{IIb}\beta_3$ are available and they permit precise in situ analysis of receptor function. Other antibodies which inhibit or enhance (13, 14) receptor function can be used for $\alpha_{IIb}\beta_3$ ligand binding studies.

In the present work, we examined the effects of truncations of $\alpha_{IIb}\beta_3$ cytoplasmic domains on initiation of cell spreading and focal adhesion formation. We found that the β subunit cytoplasmic domain is necessary and sufficient for these cytoskeleton-related processes and for recruitment of integrins to existing focal adhesions. Further, the β_3 cytoplasmic domain was also required for the contraction of fibrin clots, i.e., for the transmission of contractile events to a model extracellular matrix. In contrast, the α subunit cytoplasmic domain, while not required for initiation, regulates the integrin specificity of recruitment to focal adhesions.

Materials and Methods

Cell Culture

Clonal CHO cell lines transfected with human α_{IIb} and β_3 cDNAs or mutants $\alpha_{IIb}\beta_3$(D119→Y), $\alpha_{IIb}\beta_3(\Delta728)$, and $\alpha_{IIb}(\Delta996)\beta_3$ in pCDM8(2) vector were constructed and characterized as described (28, 38-40). The $\alpha_{IIb}(\Delta996)$ was produced by introduction of a TAA stop codon immediately downstream of the conserved GFFKR sequence of the α_{IIb} cytoplasmic domain in the CD2b plasmid (39). HT 1080 Human fibrosarcoma cells were from the American Type Culture Collection (Rockville, MD). Cells were maintained in DME (Whittaker Bioproducts Inc., Walkersville, MD), supplemented with 10% FCS (Gibco BRL Life Technologies, Inc., Gaithersburg, MD), nonessential amino acids (Sigma Immunochemicals, St. Louis, MO), and penicillin and streptomycin (Sigma Immunochemicals).

1 d after passage, cells were harvested and 10^7 cells/ml were electroporated (360 V, Capacitance = 960 μF, R4) in the presence of 20 μg DNA using a BTX 600 Electro Cell Manipulator (BTX, Inc., San Diego, CA.). After 48 h, cells were harvested and levels of $\alpha_{IIb}\beta_3$ expression determined by flow cytometry as described below. All results described herein were observed on at least three different transfections for each $\alpha\beta$ pair. In addition, stable CHO cell lines bearing $\alpha_{IIb}\beta_3$, $\alpha_{IIb}\beta_3$(D119Y), $\alpha_{IIb}\beta_3(\Delta728)$, and $\alpha_{IIb}(\Delta996)\beta_3$ were also used to further confirm results with these recombinant integrins.

Antibodies

Mouse mAb 2G12, which binds to $\alpha_{IIb}\beta_3$ (63) was from Dr. Virgil A. Woods (University of California, San Diego, CA). Rat mAb against α_5 integrin subunit Ab 16 (1) was from Dr. Kenneth M. Yamada (National Institute of Dental Research, Bethesda, MD) and the mouse anti-human anti-$\alpha_v\beta_3$ complex LM609 (10) from Dr. David A. Cheresh (The Scripps Research Institute). PAC-1 antibody against activated form of $\alpha_{IIb}\beta_3$ was from Dr. Sanford J. Shattil (54). The mAbs 15, 62 and anti-LIBS1 against β_3 (13) and mAb PL98DF6 against α_{IIb} (64) have been characterized earlier. Mouse anti-human α_2, mAb R2-8C8, specifically reacted with CHO cells transfected with human α_2 integrin subunit and immunoprecipitates $\alpha_2\beta_1$ (Faull, R., Y. Takada, and M. H. Ginsberg, unpublished results). IgG was purified from ascites fluid by using protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ). Rabbit antiserum against human platelet talin (12) was from Dr. Keith Burridge (University of North Carolina, Chapel Hill, NC).

Ligand Binding and Cell Adhesion Assays

Soluble ^{125}I-Fg or ^{125}I-anti-LIBS1 IgG binding was performed as described (39) and the ^{125}I-Fg binding results were analyzed using the LIGAND program (34). To quantify cell adhesion, cellular acid phosphatase-based (46) detection system was used. Immulon® 2 96-well plates (Dynatech laboratories, Chantilly, VI) were coated overnight at 4°C with different concentrations of fg. Cells were detached with 1 mg/ml TPCK trypsin (Worthington Biochemicals, Freehold, NJ), 3.5 mM EDTA in PBS (140 mM NaCl, 10 mM Na-phosphate, pH 7.4), washed once with 0.5 mg/ml Soy bean trypsin inhibitor (Sigma Immunochemicals) and washed twice with incubation buffer: 137.5 mM NaCl, 12 mM $NaHCO_3$, 2.6 mM KCl, 5 mM Hepes, 5 mM glucose, 2 mM $CaCl_2$, 0.1% BSA. If indicated, the LM609 ascites (final dilution 1:1,000) was first added to the wells followed by 0.4×10^5 cells/well (final volume 100 μl). After incubation the cells were washed two times with the incubation buffer and 100 μl of 6 mg/ml *p*-nitrophenyl phosphate (Sigma Immunochemicals), 1% Triton X-100, 50 mM $NaCH_3COO$, pH 5.0, was applied for 1 h at 37°C. Then, 50 μl of 1 M NaOH was added and absorbance was read at 415 nm. The linearity of the enzymatic assay was routinely verified. The points represent means of triplicate experiments ±SEM.

Microscopy and Flow Cytometry

Cell spreading assays and immunofluorescence staining were done on 12-mm circular glass coverslips (No. 1; Fisher Scientific Co., Pittsburgh, PA). The coverslips were coated overnight at 4°C with 20 μg/ml of fg fibronectin in PBS or with 30 μg/ml type 1 collagen (Nirai Gelatin Co., Osaka, Japan) in 0.05% acetic acid, followed by blocking with 1% BSA (RIA grade, Sigma Immunochemicals). The cells were detached with 0.5 g/liter trypsin, 0.5 mM EDTA (Irvine Scientific, Santa Ana, CA), washed with DME containing 10% FCS, washed twice with the same medium without serum, and seeded on the coverslips in serum-free medium for indicates times. CHO cells were fixed with 2% paraformaldehyde, 0.5% Triton X-100 for 10 min on ice, and HT1080 cells with methanol for 10 min at −20°C. The number of spread cells was counted under a phase contrast microscope by two independent observers. Flattened cells with regular margin were defined as spread. Data are reported as mean percent of spread cells from four high power microscopic fields (total number of counted cells >300). In some experiments, cell lines expressing $\alpha_{IIb}\beta_3$ or $\alpha_{IIb}\beta_3(\Delta728)$ were allowed to adhere on fibronectin-coated coverslips for 90 min and then 200 μM GRGDSP peptide (Peninsula Laboratories, Inc., Belmont, CA) was added to the media. After different time points cells were fixed and stained with anti-α_{IIb} (PL98DF6). The percentage of cells in which $\alpha_{IIb}\beta_3$ was at focal adhesions was estimated by fluorescence microscopy. At each time point >150 cells were counted. All experiments were repeated at least three times.

For immunofluorescence staining of the fixed cells, coverslips were incubated 30 min with the primary antibody in PBS, washed twice with PBS and incubated for another 30 min with FITC-conjugated goat anti-mouse $F(ab')_2$ (Tago Inc., Burlingame, CA) FITC-conjugated goat anti-rabbit IgG (Sigma Immunochemicals). For double staining the specimens were treated with irrelevant mouse IgG and then stained with biotin-labeled PL98DF6 followed by TRITC-coupled streptavidin (Molecular Probes, Inc., Eugene, OR). The coverslips were then washed and mounted in FITC-Guard™ mounting media (Testog Inc., Chicago, IL). The specimens were examined with a Leitz Orthoplan microscope with plan Apochromat 100× oil immersion objective and photographs were taken on Kodak Tmax 400 film (Eastman Kodak Co., Rochester, NY). As controls, specimens were stained with irrelevant antibodies or primary antibody was omitted.

For flow cytometry analysis of $\alpha_{IIb}\beta_3$ expression or activation status, the cells were stained in suspension for 30 min at 22°C with saturating concentrations of purified mAb 2G12 IgG or PAC-1 ascites. After washing, cells were then incubated with FITC-conjugated anti-mouse IgG or IgM (Tago Inc.) for 30 min, and examined with FACS-Scan flow cytometer (Becton-Dickinson, Lincoln Park, NJ) as described (39).

Fibrin Clot Contraction

These assays were performed by a modification of published methods (30, 36). Briefly, 3×10^6 cells in 350 μl of DME containing 25 mM Hepes were added to 10 × 75 mm glass tubes (Fisher Scientific Co.). 200 μl of pooled human platelet poor fibronectin-depleted plasma anticoagulated with 0.38% Na Citrate was added followed by 200 μl of Hepes-DME containing 28mM $CaCl_2$ and 5 U/ml human thrombin (Sigma Immunochemicals). The tubes were subsequently incubated at 37°C for 2 h and clot retraction was estimated visually.

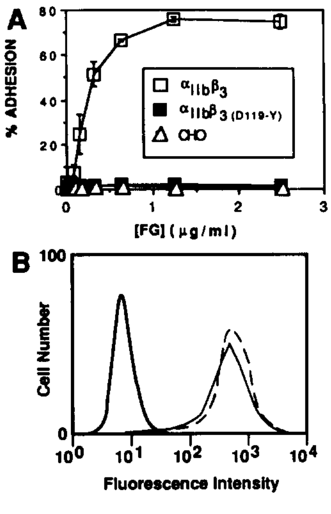

Figure 1. (*A*) Adhesion of CHO cells to fg. Cells were allowed to adhere for 30 min at 37°C and adhesion was quantified as described in Materials and Methods. Stable $a_{IIb}\beta_3$-bearing CHO cell lines (□) adhered readily, but parental CHO cells (△) or cells bearing fg-binding deficient mutant $\alpha_{IIb}\beta_3$(D119→Y) (■) failed to adhere. (*B*) Expression of wild-type and mutant $\alpha_{IIb}\beta_3$ in CHO cells. On the same day of the adhesion experiment performed in *A*, cells bearing $\alpha_{IIb}\beta_3$ (*fine solid line*) or $\alpha_{IIb}\beta_3$(D119→Y) (*dashed line*) were stained with an anti-$\alpha_{IIb}\beta_3$ (2G12). Surface expression levels were estimated by flow cytometry. Both recombinant $\alpha_{IIb}\beta_3$s were expressed at similar levels. CHO cells were negative (*bold solid line*).

Results

The α Subunit Cytoplasmic Domain Is Not Required for Initiation of Spreading and Focal Adhesion Formation

To study the role of the cytoplasmic domains in integrin function, we used CHO cells transfected with human $\alpha_{IIb}\beta_3$ integrin. Transfection of $\alpha_{IIb}\beta_3$ resulted in de novo acquisition of adhesion to fg (Fig. 1 *A*). Moreover, this adherence was dependent on the expression of functional $\alpha_{IIb}\beta_3$, since $\alpha_{IIb}\beta_3$(D119→Y), which lacks fg binding function (28), failed to mediate adhesion (Fig. 1 *A*), even though it was expressed at the same level as $\alpha_{IIb}\beta_3$ (Fig. 1 *B*). Furthermore, the $\alpha_{IIb}\beta_3$-bearing cells were able to spread on fg, and $\alpha_{IIb}\beta_3$ was localized at focal adhesions (Fig. 2 *a*). The identity of these focal adhesions was confirmed by the presence of talin (Fig. 2 *b*). Thus, CHO cell adhesion, spreading, and focal adhesion formation on Fg was dependent on the transfected integrin.

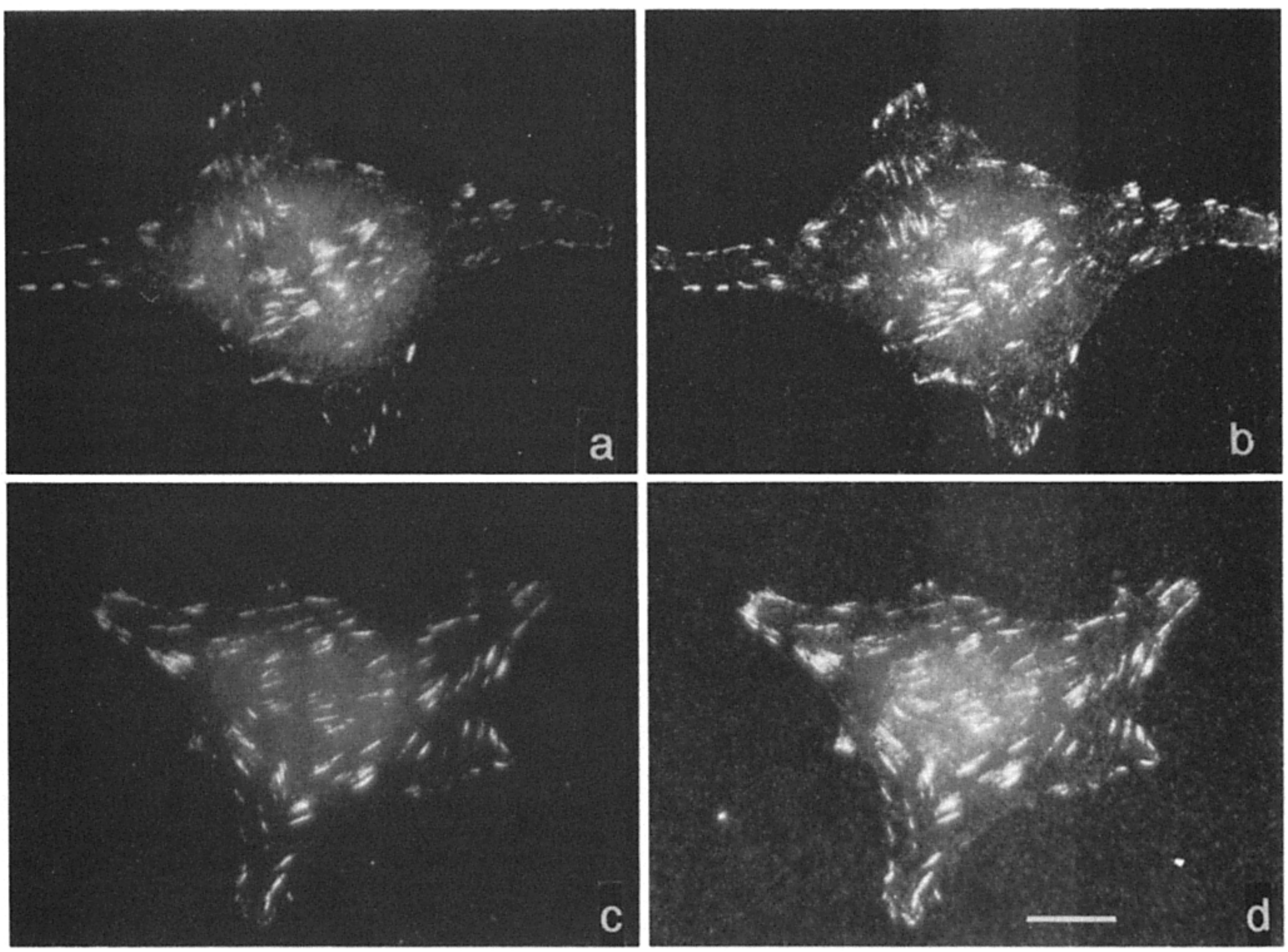

Figure 2. Identification of $\alpha_{IIb}\beta_3$ and $\alpha_{IIb}(\Delta996)\beta_3$ in focal adhesions. Stably transfected cells were permitted to adhere to fg for 2 h at 37°C. Double label immunofluorescence staining of $\alpha_{IIb}\beta_3$ (*a* and *b*) and $\alpha_{IIb}(\Delta996)\beta_3$-bearing cells (*c* and *d*) with biotinylated PL98DF6 (anti-α_{IIb}) (*a* and *c*) and polyclonal anti-talin antibodies (*b* and *d*) is depicted. Note that both the cells are well spread and α_{IIb} reactivity is found at focal adhesions identified by talin immunoreactivity. In control staining with irrelevant biotinylated antibodies, no colocalization was detected (not shown). Bar, 10 µm.

Cells bearing the α subunit truncation mutant $\alpha_{IIb}(\Delta996)\beta_3$, spread on fg and the truncated α subunit was localized at focal adhesions (Fig. 2, *c* and *d*). This suggests that the bulk of the α_{IIb} cytoplasmic domain is not required for initiation of spreading and focal adhesion formation. CHO cells express low levels of endogenous α_v, which might complex with transfected β_3. To rule out any contribution of α_v(hamster)β_3(human) heterodimers to initiation of spreading and focal adhesions, we exploited the reactivity of mAb LM609 with such heterodimers. Cells transfected with only human β_3 reacted with mAb LM609 (not shown) and acquired the capacity to adhere to fg. LM609 completely inhibited the adhesion of cells transfected with β_3 alone, whereas it had no effect on the adhesion of $\alpha_{IIb}(\Delta996)\beta_3$-bearing cells (Fig. 3 *a*). Furthermore, the $\alpha_{IIb}(\Delta996)\beta_3$-bearing cells were able to spread and form anti-α_{IIb} reactive focal adhesions in the presence of the mAb LM609 (Fig. 3, *b* and *c*). This shows that the $\alpha_{IIb}(\Delta996)\beta_3$ complex initiates cell adhesion, spreading, and focal adhesions on fg. Thus, the bulk of α_{IIb} subunit cytoplasmic tail is not needed for these processes.

Deletion of the α Subunit Cytoplasmic Domain Causes Indiscriminate Recruitment of $\alpha_{IIb}\beta_3$ to Focal Adhesions Formed by Other Integrins

To study the recruitment of the α_{IIb} truncation mutant to focal contacts we cultured cells bearing it or wild-type α_{IIb} on fibronectin. In contrast to $\alpha_{IIb}\beta_3$ (Fig. 4 *a*), $\alpha_{IIb}(\Delta996)\beta_3$ (Fig. 4 *c*) was localized at focal adhesions on fibronectin. β_1 (Fig. 4 *d*) and α_5 (not shown) immunoreactivities colocalized with that of $\alpha_{IIb}(\Delta996)$. This organization was not dependent on $\alpha_{IIb}(\Delta996)\beta_3$ binding to fibronectin, since even when expressed together with a ligand-binding deficient β_3 mutant, β_3(D119→Y) (28), $\alpha_{IIb}(\Delta996)$ was found at focal adhesions in cells cultured on fibronectin (Fig. 4 *e*). A similar pattern was observed when the cells were stained for β_3 (not shown). Since cotransfection of β_3 subunit is required for α_{IIb} surface expression (38), β_3 was the only β subunit associated with $\alpha_{IIb}(\Delta996)$. Thus, the staining of focal adhesions for both α_{IIb} and β_3 shows that $\alpha_{IIb}(\Delta996)\beta_3$(D119→Y) could be recruited to focal adhesions formed by $\alpha_5\beta_1$ in spite of a profound defect in ligand binding.

Since recruitment to focal adhesions is a feature of ligand-bound integrins (27), we compared the reactivity of $\alpha_{IIb}\beta_3$ and $\alpha_{IIb}(\Delta996)\beta_3$ with mAbs specific for the ligand-occupied or activated forms of $\alpha_{IIb}\beta_3$. Either with anti-LIBS1 (Fig. 5 *A*) or PAC-1 (Fig. 5 *B*) antibodies, only a low basal reactivity was detected. The anti-LIBS1 and PAC-1 binding was dramatically increased by the appropriate inducing agents (RGDS peptide or activating antibody, respectively). Thus, in contrast to the previously characterized α_{IIb} subunit cytoplasmic mutations (40), the $\alpha_{IIb}(\Delta996)$ truncation did not result in detectable conformational changes of the extracellular domain of the receptor.

To determine whether recruitment of $\alpha_{IIb}(\Delta996)$ to focal adhesions is specific for CHO cells, for fibronectin substrata, or for focal adhesions formed by $\alpha_5\beta_1$, we transiently transfected $\alpha_{IIb}\beta_3$ or $\alpha_{IIb}(\Delta996)\beta_3$ into HT1080 human fibrosarcoma cells. These cells express $\alpha_5\beta_1$ and $\alpha_2\beta_1$ and spread on both fibronectin and collagen. On fibronectin, $\alpha_5\beta_1$ was detected at focal adhesions (Fig. 6 *g*) and on collagen $\alpha_2\beta_1$ (Fig. 6 *f*) was in the focal adhesions. On both of these substrates, $\alpha_{IIb}(\Delta996)$ (Fig. 6, *a* and *b*) but not wild-

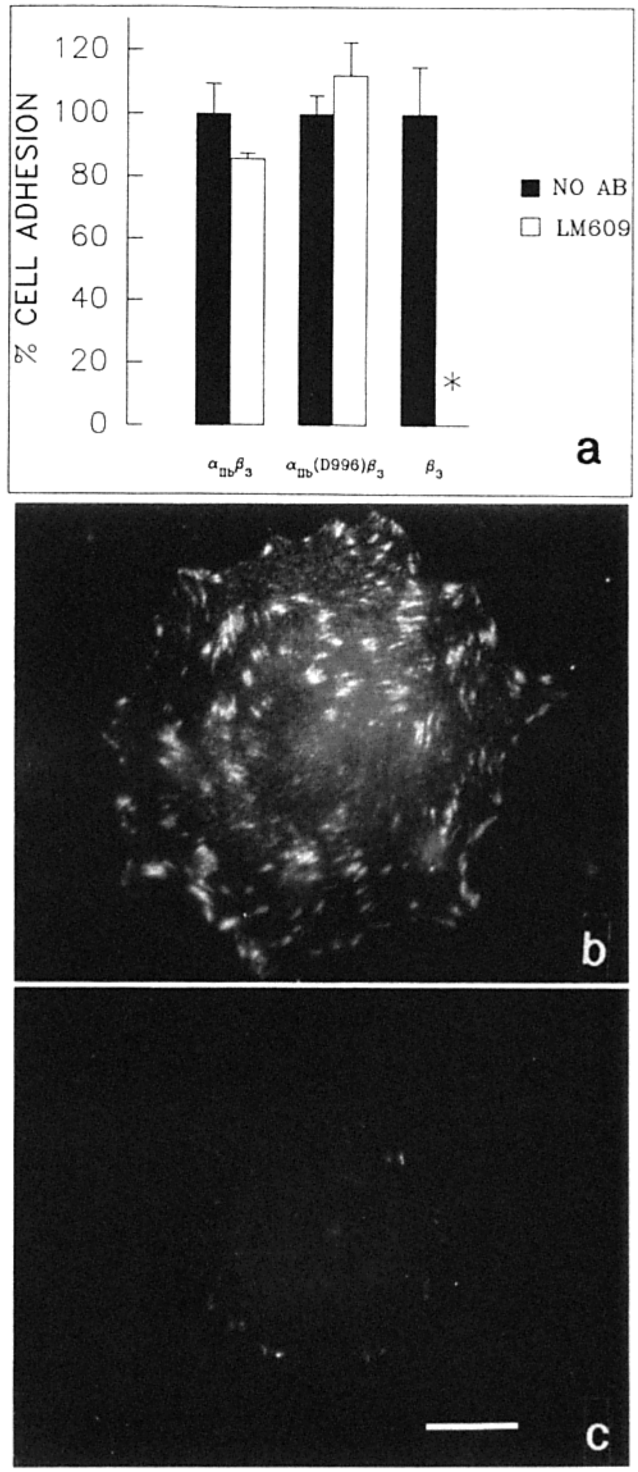

Figure 3. $\alpha_{IIb}(\Delta996)\beta_3$ initiates spreading and focal adhesion formation (*a*). Cells were allowed to adhere for 30 min at 37°C to plates coated with 5 μg/ml of fg. mAb LM609 had no effect on the adhesion of stable $\alpha_{IIb}\beta_3$ or $\alpha_{IIb}(\Delta996)\beta_3$-bearing cell lines, but completely inhibited the adhesion of cells transfected with human β_3 alone (* no adhesion detected). In each experiment adhesion in the absence of the antibody was taken as 100% value (*b* and *c*). When $\alpha_{IIb}(\Delta996)\beta_3$-bearing cells were allowed to adhere on fg for 2 h in the presence of LM609, they were completely spread and α_{IIb} was detected at focal adhesions (*b*, mAb PL98DF6). The reactivity was due to the anti-α_{IIb}, since when anti-α_{IIb} was omitted, no staining of focal adhesions was observed (*c*). Bar, 10 μm.

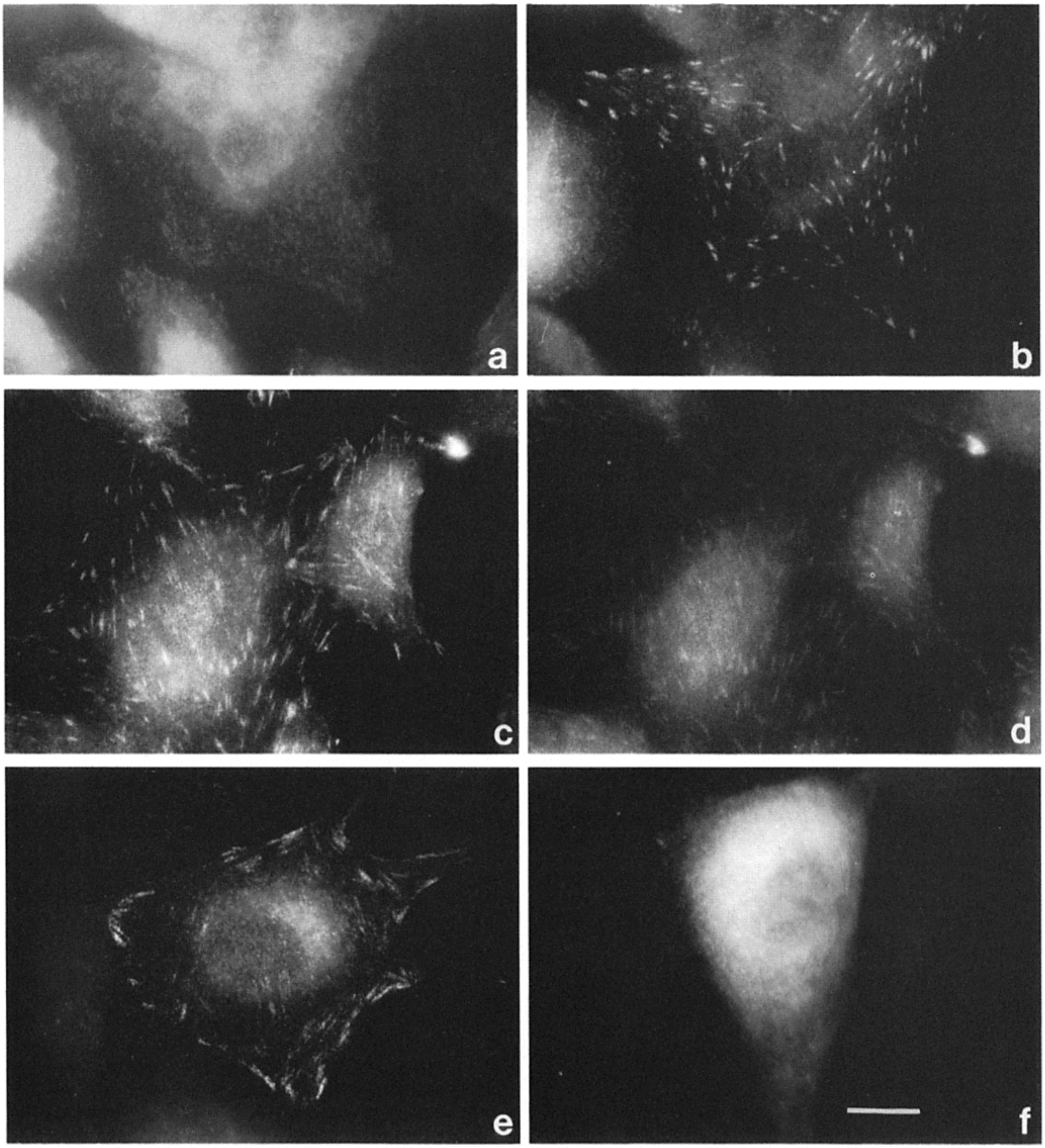

Figure 4. Ligand-binding independent recruitment of $\alpha_{IIb}(\Delta996)\beta_3$ to focal adhesions. CHO cells were transfected and after 48 h were cultured on fibronectin substrates for 2 h. The cells were then stained with biotinylated anti-human α_{IIb} (mAb PL98DF6) (*a*, *c*, *e*, and *f*) or anti-hamster β_1 (7E2) (*b* and *d*). The same cell was photographed in *a*, *b*, and *c*, *d*. The transfected integrins were $\alpha_{IIb}\beta_3$ (*a* and *b*), $\alpha_{IIb}(\Delta996)\beta_3$ (*c* and *d*), $\alpha_{IIb}(\Delta996)\beta_3$(D119→Y) (*e*), and $\alpha_{IIb}\beta_3$(D119→Y) (*f*). $\alpha_{IIb}(\Delta996)$ (*c*), but not wild-type α_{IIb} (*a*), was detected at focal adhesions containing hamster β_1. Ligand binding defective $\alpha_{IIb}(\Delta996)\beta_3$(D119→Y) was detected at focal adhesions in cells cultured on fibronectin (*e*), while $\alpha_{IIb}\beta_3$(D119→Y) had a uniform cell surface distribution (*f*).

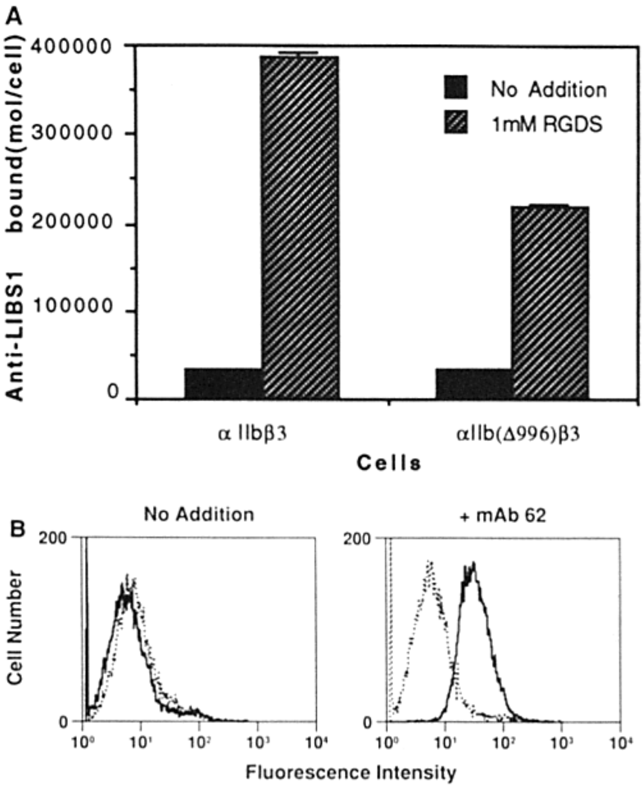

Figure 5. (*A*) Lack of spontaneous anti-LIBS1 binding to $\alpha_{IIb}(\Delta996)\beta_3$. CHO cells bearing $\alpha_{IIb}(\Delta996)\beta_3$ or $\alpha_{IIb}\beta_3$ were incubated with 275 nM ^{125}I-anti-LIBS1 in the presence (▨) or absence (■) of 1 mM RGDS peptide. Anti-LIBS1 binding was estimated as described in Materials and Methods and results were expressed as molecules bound/cell ±SE of triplicates. (*B*) $\alpha_{IIb}(\Delta996)\beta_3$ is in a low affinity state. 50 μl of a suspension of 4 × 10^6 $\alpha_{IIb}(\Delta996)\beta_3$-bearing CHO cells/ml were incubated with fluorescein-conjugated PAC1 in the presence (*dotted line*) or absence (*solid line*) of 1 mM RGDS peptide. In the right panel, the cells were stimulated with 2 μM mAb 62. After 20 min at room temperature, the suspension was diluted with 450 μl Tyrode's buffer and PAC1 binding was measured by flow cytometry as described (39).

type α_{IIb} (Fig. 6, *c* and *d*) was detected at focal adhesions. This shows that the recruitment of $\alpha_{IIb}(\Delta996)$ to focal adhesions is not specific to CHO cells and it is not dependent on the integrin forming the focal adhesions. Furthermore this provides additional evidence that this recruitment does not depend on ligand binding, since $\alpha_{IIb}\beta_3$ does not bind directly to collagen with high affinity (50).

Integrin β_3 Subunit Cytoplasmic Domain Is Necessary for Cell Spreading and Initiation of Focal Adhesions but not for Ligand Binding

Only 0.6 ± 0.4% of cells expressing α_{IIb} with a β_3 subunit truncation mutant $\beta_3(\Delta728)$, spread within one h of plating on fg. In contrast, 83 ± 1.4% of cells bearing wild-type $\alpha_{IIb}\beta_3$ were spread. To find out whether $\beta_3(\Delta728)$-bearing cells failed to spread on fg because of defects on ligand binding or because of defects in post-occupancy events in cell adhesion, we measured the capacity of the $\alpha_{IIb}\beta_3(\Delta728)$-bearing cells to bind soluble fg and to adhere to fg-coated surfaces. After activation with mAb62 (39), $\alpha_{IIb}\beta_3(\Delta728)$-bearing cells bound fg with an affinity constant, Ka, of 7.92*10^6 ± 0.96*10^6 M^{-1} (K_d = 126 nM). This was not significantly different from the affinity constant for $\alpha_{IIb}\beta_3$-bearing cells (9.1*10^6 ± 1.5*10^6 M^{-1}, K_d = 110 nM) (39). Thus, the cytoplasmic truncation of β_3 did not alter the affinity of activated $\alpha_{IIb}\beta_3$ for soluble fg.

In short-term adhesion assays, when either the $\alpha_{IIb}\beta_3$ or $\alpha_{IIb}\beta_3(\Delta728)$-bearing cells were not spread (Fig. 7, *b* and *d*), both cells adhered equally well on fg (Fig. 7 *a*, 20 min). After a 1-h incubation at 37°C, when the $\alpha_{IIb}\beta_3$-bearing cells were spread (Fig. 7 *e*), they adhered better (Fig. 7 *a*, 1 h) than $\alpha_{IIb}\beta_3(\Delta728)$-bearing cells which were not spread (Fig. 7 *c*). Together with the data that the β_3 subunit truncation does not affect the fg binding affinity of $\alpha_{IIb}\beta_3$, this shows that its initial interaction with fg is similar to the wild-type receptor. In contrast, because of the inability of $\alpha_{IIb}\beta_3(\Delta728)$ to provoke post-occupancy events, the long-term adhesion mediated by $\alpha_{IIb}\beta_3(\Delta728)$ is less stable than that mediated by wild-type $\alpha_{IIb}\beta_3$.

The β_3 Cytoplasmic Domain Is Required for Recruitment to Existing Focal Adhesions

To study the recruitment of $\alpha_{IIb}\beta_3$ and $\alpha_{IIb}\beta_3(\Delta728)$ to existing focal adhesions, we allowed CHO cells expressing these complexes to spread on fibronectin and form complexes containing $\alpha_5\beta_1$. The redistribution of α_{IIb} to these focal adhesions was then analyzed by exploiting the findings of LaFlamme and co-workers (27) that soluble ligand binding promotes integrin recruitment to focal adhesions. As described above, wild-type $\alpha_{IIb}\beta_3$ was excluded from the focal adhesions formed under these conditions. After addition of GRGDSP peptide, $\alpha_{IIb}\beta_3$ was localized at focal adhesions in 80% of the cells. In contrast, <10% of cells had $\alpha_{IIb}\beta_3(\Delta728)$ at focal adhesions before or after peptide addition (Fig. 8). Thus, $\alpha_{IIb}\beta_3$ is recruited to focal adhesions after addition of soluble ligands and this recruitment is dependent on the β subunit cytoplasmic domain.

The β_3 Cytoplasmic Domain Is Required for Contraction of Fibrin Gels

The foregoing experiments strongly implicate the β_3 cytoplasmic domain in linkage of the integrin to cytoskeleton-dependent events. To directly assess the functional significance of this potential linkage, we examined the effect of removal of this domain on the capacity of $\alpha_{IIb}\beta_3$ to mediate fibrin clot contraction. Cells transfected with $\alpha_{IIb}\beta_3$ produced ~50% reduction in the volume of fibrin clots over 2 h. In contrast, untransfected CHO cells failed to retract such clots establishing that this cytoskeleton dependent contractile event was mediated by the recombinant integrin. The β_3 truncation mutant also failed to contract the clot (Fig. 9) even though it expressed comparable levels of the recombinant integrin. Thus these data establish that the β_3 cytoplasmic domain is required for transmission of contractile force to the fibrin matrix.

Discussion

The major findings of this paper are: (*a*) the β_3 subunit cytoplasmic domain is necessary and sufficient for $\alpha_{IIb}\beta_3$-mediated cell spreading, but its truncation does not change intrinsic fg binding function; (*b*) the capacity of $\alpha_{IIb}\beta_3$ to

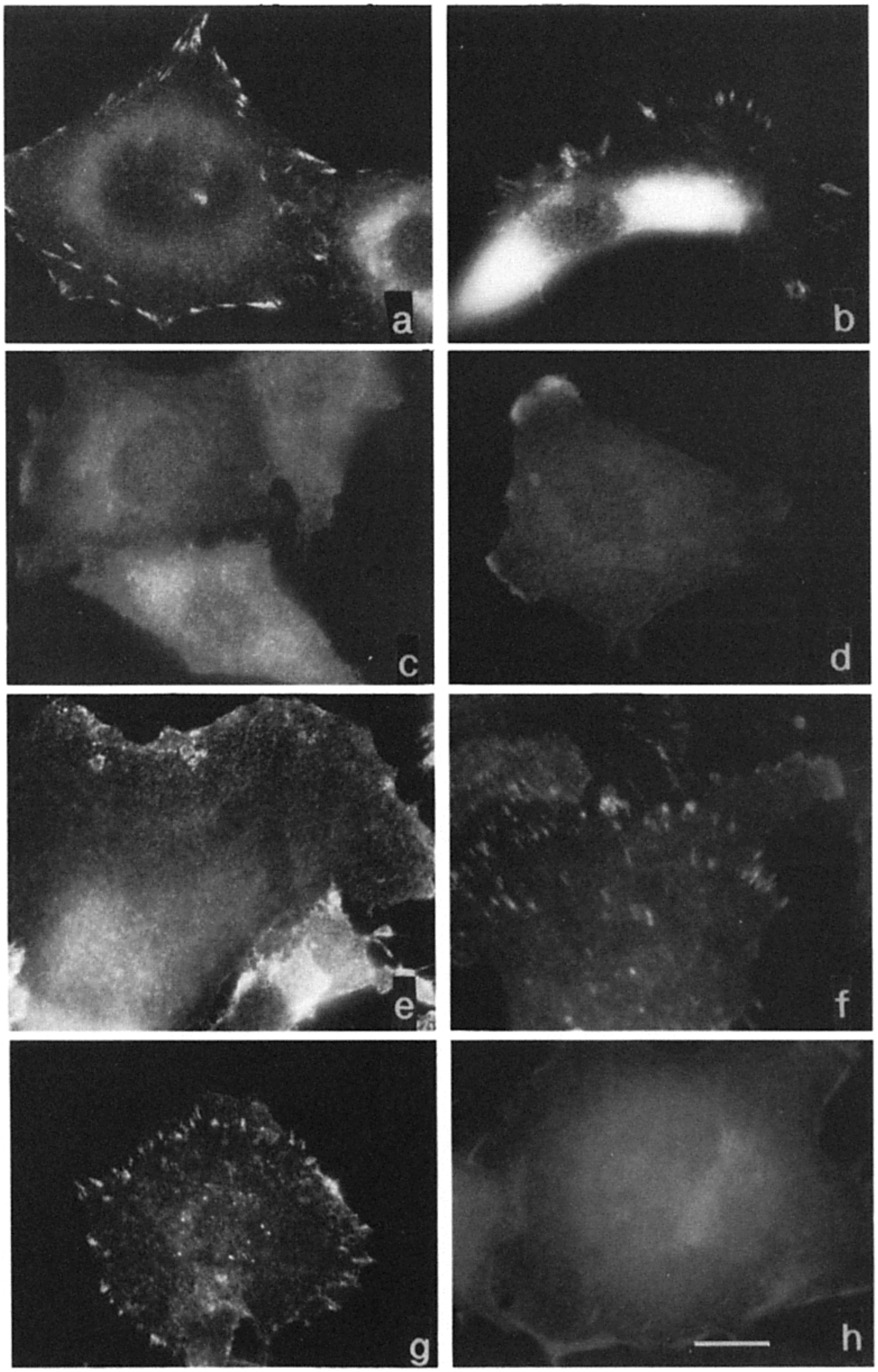

Figure 6. Ligand-independent recruitment of $\alpha_{IIb}(\Delta996)\beta_3$ to focal adhesions occurs on multiple substrates, with multiple β_1 integrins and in multiple cell types. HT1080 human fibrosarcoma cells were transiently transfected with either $\alpha_{IIb}(\Delta996)\beta_3$ (*a* and *b*) or $\alpha_{IIb}\beta_3$ (*c* and *d*). After 48 h, cells were cultured for 2 h on fibronectin (*a*, *c*, *e*, and *g*) or collagen (*b*, *d*, *f*, and *h*). Anti-α_{IIb} (PL98DF6) revealed focal adhesions in $\alpha_{IIb}(\Delta996)\beta_3$-bearing cells on both fibronectin (*a*) and collagen (*b*). In $\alpha_{IIb}\beta_3$-bearing cells no such localization was observed on either substrate (*c* and *d*). Anti-α_2 mAb (R2-8C8, *e* and *f*) stained focal adhesions in HT1080 cells cultured on collagen (*f*) but not on fibronectin (*e*). Anti-α_5 mAb (Ab 16; *g* and *h*) stained focal adhesions on fibronectin (*g*), but not on collagen (*h*).

mediate retraction of fibrin clots is dependent on the β_3 cytoplasmic domain; (*c*) the bulk of the α_{IIb} subunit cytoplasmic domain is not needed for initiation of cell spreading and focal contact formation by $\alpha_{IIb}\beta_3$; and (*d*) α_{IIb} subunit cytoplasmic domain truncation allows ligand independent integrin recruitment to focal adhesions formed by other integrins. Thus, the α subunit cytoplasmic domain serves to limit integrin recruitment to existing focal adhesions.

The β_3 cytoplasmic domain was both necessary and sufficient for initiation of cell spreading and focal adhesion formation. The failure of $\alpha_{IIb}\beta_3(\Delta728)$ to provoke these processes establishes necessity. Conversely the capacity of

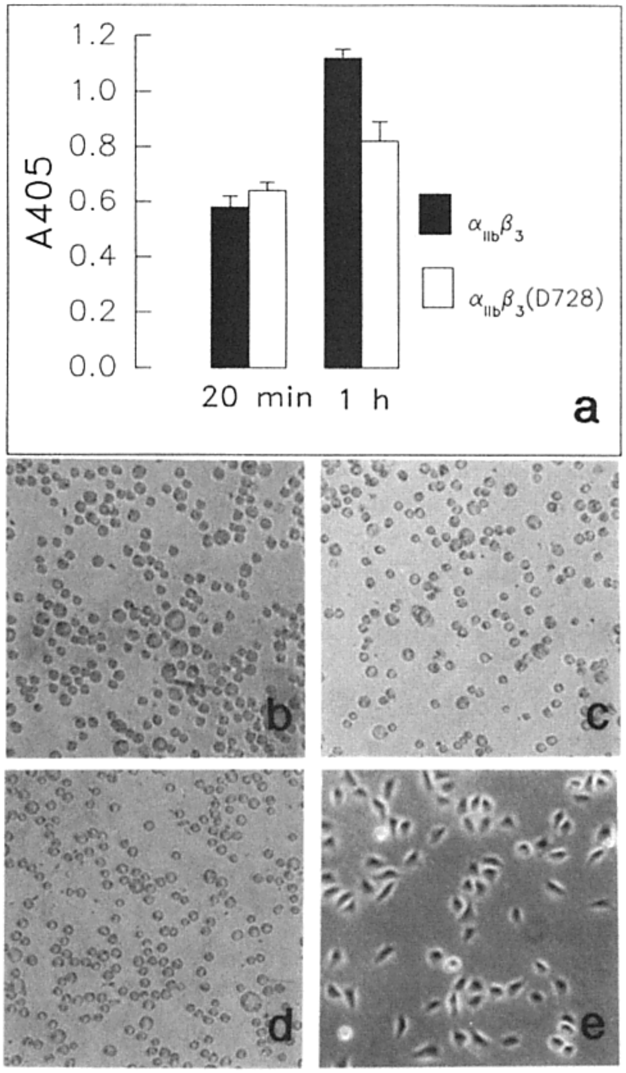

Figure 7. The β_3 cytoplasmic domain is required for initiation of cell spreading. Stable cell lines bearing $\alpha_{IIb}\beta_3$ or $\alpha_{IIb}\beta_3(\Delta728)$ adhere to fg for 20 min at 22°C or 1 h at 37°C. (*a*) Quantification of adhesion. (*b* and *c*) Phase contrast micrographs of $\alpha_{IIb}\beta_3(\Delta728)$ cells after 20 min and 1 h, respectively. (*d* and *e*) Phase contrast micrographs of $\alpha_{IIb}\beta_3$ cells after 20 min and 1 h, respectively. $\alpha_{IIb}\beta_3(\Delta728)$-bearing cells were not spread in either conditions. $\alpha_{IIb}\beta_3$-bearing cells were not spread at 20 min (*d*) but were spread after 1 h (*e*).

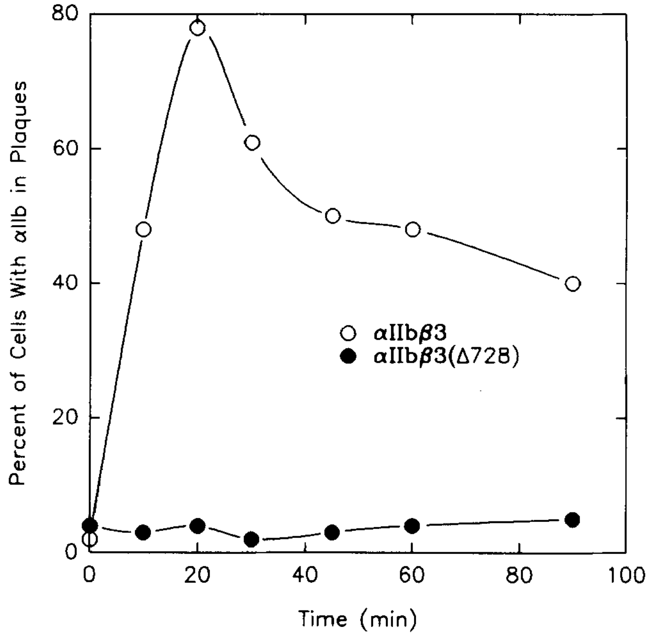

Figure 8. The β_3 subunit cytoplasmic domain is required for recruitment to focal adhesions. Cells stably transfected with $\alpha_{IIb}\beta_3$ or $\alpha_{IIb}\beta_3(\Delta728)$ were cultured on a fibronectin substratum for 90 min. After addition of 200 μM GrGDSP, the cells were fixed at the indicated time and the cells were stained with anti-α_{IIb} (PL98DF6). The percentage of cells (total = 200 for each time point) containing at least 1 α_{IIb}-containing focal adhesion was enumerated. In the absence of peptide, <10% of cells contained α_{IIb}-positive focal adhesions at each time point. Addition of GRGDSP provoked a prompt redistribution of wild-type $\alpha_{IIb}\beta_3$ to focal adhesions. The percentage of cells with $\alpha_{IIb}\beta_3(\Delta728)$ at focal adhesions did not change after peptide addition.

$\alpha_{IIb}\beta_3$ that lacked most of the α_{IIb} cytoplasmic domain to support these processes establishes sufficiency with respect to integrin cytoplasmic elements. These activities of $\alpha_{IIb}\beta_3$ may be related to its capacity to stimulate Na^+-H^+ exchange (3) or activate tyrosine kinases (15). Indeed, the β_1 cytoplasmic domain is similar to that of β_3 and is involved in both of these signaling events (17, 53). In addition, based on similar logic, the β_3 cytoplasmic domain was necessary and sufficient for recruitment to existing focal adhesions. The β_1 and β_3 cytoplasmic domains are interchangeable for localization to focal adhesions (57). Moreover, chimeras containing the cytoplasmic domain of β_1 linked to the IL-2 receptor (Tac) (27) or to N-Cadherin (49) are recruited to focal adhesions. Thus, these findings may be applicable to many integrin classes. Studies using purified integrins and synthetic peptides from the cytoplasmic domain of β_1 integrin subunit have shown that specific interaction sites for cytoplasmic focal adhesion proteins talin and α-actinin reside in the β_1 subunit cytoplasmic domain (22, 41). The binding of β subunit cytoplasmic domains to cytoplasmic focal adhesion proteins would provide a cogent mechanism for recruitment. The β_1 cytoplasmic sequences involved in localization to focal adhesions have been mapped (19, 31, 47, 56) and at least one site resides within an α-actinin binding motif (47). In addition to α-actinin binding motif, β_3 shares motifs with several other β subunit cytoplasmic domains. In particular, there is an NPXY(F) sequence which may form a tight turn (9) and mediate internalization of cell surface receptors. $\alpha_{IIb}\beta_3$ is internalized and mediates the endocytosis of fg and its storage in secretion granules (18, 62), possibly via the NPXY(F) motif. Moreover, mutations in these motifs inhibited localization of β_1 integrins (47) and the adhesive function of a β_2 integrin (20). Similarly, the β_3 cytoplasmic domain shares the triplet of hydroxylated residues involved in the adhesive function of $\alpha_L\beta_2$(20). The fine mapping of the sites in the cytoplasmic domain of β_3 involved in initiation of spreading, and comparison with the sites involved in recruitment, signaling, and internalization, should elucidate the hierarchy of these events.

In spite of the role of β_3 subunit cytoplasmic domain in cell spreading and focal adhesion formation, there was no

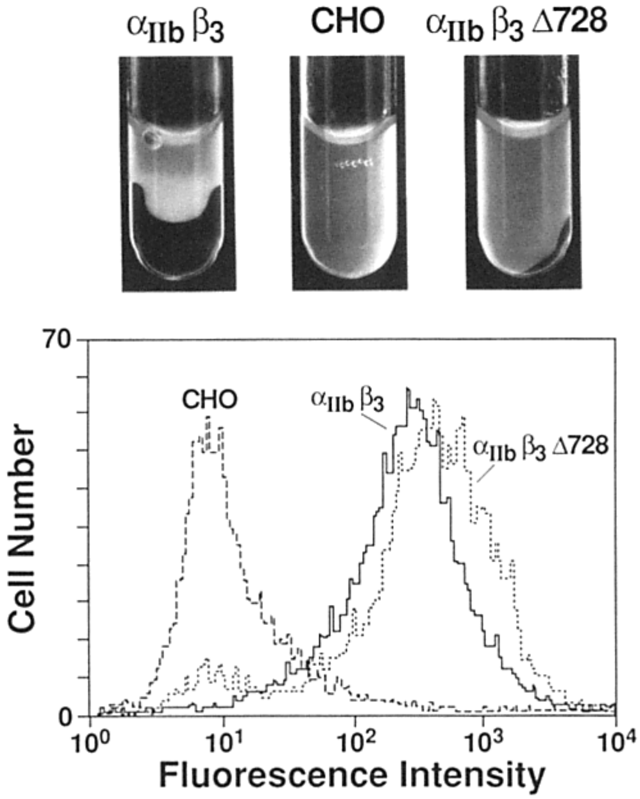

Figure 9. The β_3 cytoplasmic domain mediates fibrin clot retraction. 3×10^6 cells, bearing the indicated transfected integrin, were mixed with 200 μl of pooled plasma and 200 μl of Hepes-DME containing 28 mM $CaCl_2$ and 5 U/ml human thrombin in a total volume of 750 μl. The tubes were subsequently incubated at 37°C for 2 h and clot retraction was estimated visually. The top panel depicts photographs of the clots and the bottom panel FACS histograms of the same cells stained with an anti-$\alpha_{IIb}\beta_3$, 2G12.

quantitative defect in ligand binding to $\alpha_{IIb}\beta_3$ that lacked nearly all of the β_3 cytoplasmic domain. First, $\alpha_{IIb}\beta_3(\Delta728)$ mediated the binding of soluble fg with a similar affinity to wild-type $\alpha_{IIb}\beta_3$. These experiments necessitated the use of mAb62 to induce soluble fg binding and it is possible that the mAb could have overcome a quantitative defect. Nevertheless, in the absence of mAb62, $\alpha_{IIb}\beta_3(\Delta728)$ was comparable with the wild-type receptor in its ability to promote initial adhesion to fg. A defect in the ability of $\alpha_{IIb}\beta_3(\Delta728)$ to mediate cell adhesion was apparent after the wild-type $\alpha_{IIb}\beta_3$-bearing cells were allowed to spread. Since cell spreading stabilizes cell adhesion, the defect in the adhesion of $\alpha_{IIb}\beta_3(\Delta728)$-bearing cells is due to a lack of stabilization. it is possible that failure of stabilization might account for the defects in cell adhesion caused by mutations of the β_1 or β_2 integrin cytoplasmic domains (19, 21).

An important finding is that the α subunit cytoplasmic domain plays a central role in limiting recruitment of integrins to focal adhesions. It is clear that there is remarkable specificity in recruitment of integrins to focal adhesions (11, 12). This specificity was manifest in these experiments by the failure of $\alpha_{IIb}\beta_3$ to enter focal adhesions formed during adhesion to fibronectin. The recombinant $\alpha_{IIb}\beta_3$ in CHO cells does not enter the $\alpha_5\beta_1$ focal adhesions because it is in a resting state (39) and therefore does not bind fibronectin with high affinity (45) nor mediate cell adhesion to fibronectin (26, 51). Truncation of the α_{IIb} cytoplasmic domain removed the constraint on entry of $\alpha_{IIb}\beta_3$ into focal adhesions. The effect of truncation was not due to activation of the ligand binding function of $\alpha_{IIb}\beta_3$ because: (*a*) $\alpha_{IIb}(\Delta996)\beta_3$ was not activated as judged by PAC1 binding; (*b*) $\alpha_{IIb}(\Delta996)\beta_3$ entered focal adhesions formed on collagen even though $\alpha_{IIb}\beta_3$ apparently does not bind to collagen (50); and (*c*) $\alpha_{IIb}(\Delta996)\beta_3$, bearing a point mutation which abrogates measurable ligand binding, was recruited to focal adhesions formed on fibronectin. These experiments suggest that the α subunit cytoplasmic domain inhibits interactions of the β subunit cytoplasmic domain with cytoskeletal elements and thus constrains recruitment to focal adhesions. Ligand binding to integrins relieves this constraint (27). In support of this, electron microscopic images of purified integrins (6, 35, 60) show that the relationships of the strands containing the transmembrane domains are quite variable and a suggestion that ligand binding might influence this relationship (60). It is possible that there might also be α subunit-specific recruitment of unoccupied integrins to focal adhesions. This is based on the wide (140 nm) spacing required for ligands to initiate focal adhesions (33). At this spacing, unoccupied integrins could readily pack with occupied integrins. Moreover, ligand binding defective $\alpha_5\beta_1$ is recruited to focal adhesions formed by wild-type receptor (58). It seems likely that the process of recruitment may be a complex function of integrin interactions on both faces of the plasma membrane as well as potential lateral interactions between integrins.

The present work has centered on focal adhesions and cell spreading, in vitro correlates of integrin-dependent cytoskeletal reorganization. Contraction of extracellular matrix gels is a function of the cytoskeleton relevant to the apposition of wounds and consequent healing (52). This process is dependent on integrins (5, 37, 52) and previous work suggested that the α subunit cytoplasmic domains are involved (8). In particular, recombinant $\alpha_2\beta_1$ failed to contract collagen gels when the cytoplasmic domain of α_2 was replaced with that of α_4. The present work establishes that the β_3 subunit cytoplasmic domain is essential for the integrin-mediated transmission of contractile force to the extracellular matrix. It seems that ligand binding removes a constraint imposed by the α cytoplasmic domain on cytoskeletal linkage to the β subunit. Thus, it is possible that collagen binding to the extracellular domain of chimeric $\alpha_2\beta_1$ fails to relieve the constraint imposed by the α_4 cytoplasmic domain.

Prior studies (27, 49) and the present work suggest rules governing the integrin repertoire at focal contacts. (*a*) Integrin β_1 or β_3 cytoplasmic domains are necessary and sufficient for initiation of cell spreading and focal adhesion formation. α subunit cytoplasmic domains are not required for initiation of these processes in β_1 or β_3 integrins. (*b*) α subunit cytoplasmic domains limit recruitment by constraining interactions of the β_1 or β_3 cytoplasmic domain with components of the focal adhesions. (*c*) The constraints on recruitment imposed by the α subunit cytoplasmic domain can be eliminated by its deletion or by ligand binding to the integrin.

Integrin cytoplasmic domains influence cellular signaling elements including Tyrosine kinases, phospholipid metabolism, and ion transporters (53). Moreover, different integrins may elicit differing responses. Since focal adhesions concentrate a number of signaling molecules, they may be instrumental in integrin signaling. The repertoire of integrins assembled at focal adhesions may then specify the consequences of cell adhesion for growth, migration, and gene expression. Loss of the capacity of α subunit cytoplasmic domains to constrain recruitment through mutation, splicing variants (59), phosphorylation (42, 55), or other posttranslational modification could alter the repertoire of integrins at focal adhesions. This could dramatically alter cellular responses to positional cues provided by the extracellular matrix.

We gratefully acknowledge Drs. Virgil Woods, Sanford J. Shattil, Keith Burridge, David Cheresh, Rudolph Juliano, and Kenneth M. Yamada for the gifts of antibodies.

This work was supported by National Institutes of Health grants HL-48728, HL-28235, HL-16411, and HL-42977. J. Ylänne and T. E. O'Toole are postdoctoral fellows of the Arthritis Foundation.

Received for publication 13 November 1992 and in revised form 18 March 1993.

References

1. Akiyama, S. K., S. S. Yamada, W-T. Chen, and K. M. Yamada. 1989. Analysis of fibronectin receptor function with monoclonal antibodies: Roles in cell adhesion, migration, matrix assembly, and cytoskeletal organization. *J. Cell Biol.* 109:863–875.
2. Aruffo, A., and B. Seed. 1987. Molecular cloning of a CD28 cDNA by a high efficiency COS cell expression system. *Proc. Natl. Acad. Sci. USA.* 84:8573–8577.
3. Banga, H. S., E. R. Simons, L. F. Brass, and S. E. Rittenhouse. 1986. Activation of phospholipases A and C in human platelets exposed to epinephrine: Role of glycoproteins IIb/IIIa and dual role of epinephrine. *Proc. Natl. Acad. Sci. USA.* 83:9197–9201.
4. Burridge, K., K. Fath, T. Kelley, G. Nuckolls, and N. Turner. 1988. Focal adhesions: Transmembrane junctions between the extracellular matrix and the cytoskeleton. *Annu. Rev. Cell Biol.* 4:487–525.
5. Caen, J. P., P. A. Castaldi, J. C. Leclerc, S. Inceman, M. J. Larrieu, M. Probst, and J. Bernard. 1966. Congenital bleeding disorders with long bleeding time and normal platelet count 1. Glanzmann's Thrombasthenia (Report of Fifteen Patients). *Am. J. Med.* 41:4–26.
6. Carrell, N. A., L. A. Fitzgerald, B. Steiner, H. P. Erickson, and D. R. Phillips. 1985. Structure of human platelet membrane glycoproteins IIb and IIIa as determined by electron microscopy. *J. Biol. Chem.* 260: 1743–1749.
7. Chan, P.-Y., M.B. Lawrence, M. L. Dustin, L. M. Ferguson, D. E. Golan, and T. A. Springer. 1991. Influence of receptor lateral mobility on adhesion strengthening between membranes containing LFA-3 and CD2. *J. Cell Biol.* 115:245–255.
8. Chan, B. M. C., P. D. Kassner, J. A. Schiro, H. R. Byers, T. S. Kupper, and M. E. Hemler. 1992. Distinct cellular functions mediated by different VLA integrin α subunit cytoplasmic domains. *Cell.* 68:1051–1060.
9. Chen, W.-J., J. L. Goldstein, and M. S. Brown. 1990. NPXY, a sequence often found in cytoplasmic tails, is required for coated Pit-mediated internalization of the low density lipoprotein receptor. *J. Biol. Chem.* 265: 3116–3123.
10. Cheresh, D. A. 1987. Human endothelial cells synthesize and express an Arg-Gly-Asp-directed adhesion receptor involved in attachment of fibrinogen and von Willebrand factor. *Proc. Natl. Acad. Sci. USA.* 84: 6471–6475.
11. Dejana, E., S. Colella, G. Conforti, M. Abbadini, M. Gaboli, and P. C. Marchisio. 1988. Fibronectin and vitronectin regulate the organization of their respective Arg-Gly-Asp adhesion receptors in cultured human endothelial cells. *J. Cell Biol.* 107:1215–1223.
12. Fath, K. R., C.-J. S. Edgell, and K. Burridge. 1989. The distribution of distinct integrins in focal contacts is determined by the substratum composition. *J. Cell. Sci.* 92:67–75.
13. Frelinger, A. L., III, I. Cohen, E. F. Plow, M. A. Smith, J. Roberts, S. C.-T. Lam, and M. H. Ginsberg. 1990. Selective inhibition of integrin function by antibodies specific for ligand-occupied receptor conformers. *J. Biol. Chem.* 265:6346–6352.
14. Frelinger, A. L., III, X. Du, E. F. Plow, and M. H. Ginsberg. 1991. Monoclonal antibodies to ligand-occupied conformers of integrin $\alpha_{IIb}\beta_3$ (Glycoprotein IIb-IIIa) alters receptor affinity, specificity, and function. *J. Biol. Chem.* 266:17106–17111.
15. Golden, A., J. S. Brugge, and S. J. Shattil. 1990. Role of platelet membrane glycoprotein IIb-IIIa in agonist-induced tyrosine phosphorylation of platelet proteins. *J. Cell Biol.* 111:3117–3127.
16. Grinnell, F. 1977. Cellular adhesiveness and extracellular substrata. *Int. Rev. Cytol.* 53:65–144.
17. Guan, J-L., J. E. Trevithick, and R. O. Hynes. 1991. Fibronectin/integrin interaction induces tyrosine phosphorylation of a 120-kDa protein. *Cell Regul.* 2:951–964.
18. Handagama, P., D. A. Rappolee, Z. Werb, J. Levin, and D. F. Bainton. 1990. Platelet alpha granule fibrinogen, albumin, and immunoglobulin G are not synthesized by rat and mouse megakaryocytes. *J. Clin. Invest.* 86:1364–1368.
19. Hayashi, Y., B. Haimovich, A. Reszka, D. Boettiger, and A. Horwitz. 1990. Expression and function of chicken integrin beta 1 subunit and its cytoplasmic domain mutants in mouse NIH 3T3 cells. *J. Cell Biol.* 110:175–184.
20. Hibbs, M. L., S. Jakes, S. A. Stacker, R. W. Wallace, and T. A. Springer. 1991. The cytoplasmic domain of the integrin lymphocyte function-associated antigen 1 β subunit: sites required for binding to intercellular adhesion molecule 1 and the phorbol ester-stimulated phosphorylation site. *J. Exp. Med.* 174:1227–1238.
21. Hibbs, M. L., H. Xu, S. A. Stacker, and T. A. Springer. 1991. Regulation of adhesion to ICAM-1 by the cytoplasmic domain of LFA-1 integrin beta subunit. *Science (Wash. DC).* 251:1611–1613.
22. Horwitz, A., K. Duggan, C. A. Buck, M. C. Beckerle, and K. Burridge. 1986. Interaction of plasma membrane fibronectin receptor with talin–a transmembrane linkage. *Nature (Lond.).* 320:531–533.
23. Hynes, R. O. 1987. Integrins: a family of cell surface receptors. *Cell.* 48:549–554.
24. Hynes, R. O. 1992. Integrins: versatility, modulation, and signalling in cell adhesion. *Cell.* 69:11–25.
25. Deleted in proof.
26. Kieffer, N., L. A. Fitzgerald, D. Wolf, D. A. Cheresh, and D. R. Phillips. 1991. Adhesive properties of the β_3 integrins: comparison of GPIIb-IIIa and the vitronectin receptor individually expressed in human melanoma cells. *J. Cell Biol.* 113:451–461.
27. LaFlamme, S. E., S. K. Akiyama, and K. M. Yamada. 1992. Regulation of fibronectin receptor distribution. *J. Cell Biol.* 117:437–447.
28. Loftus, J. C., T. E. O'Toole, E. F. Plow, A. Glass, A. L. Frelinger, III, and M. H. Ginsberg. 1990. A β_3 integrin mutation abolishes ligand binding and alters divalent cation-dependent conformation. *Science (Wash. DC).* 249:915–918.
29. Lotz, M. M., C. A. Burdsal, H. P. Erickson, and D. R. McClay. 1989. Cell adhesion to fibronectin and tenascin: quantitative measurements of initial binding and subsequent strengthening response. *J. Cell Biol.* 109:1795–1805.
30. Macieira-Coelho, A., and B. Azzarone. 1990. Correlation between contractility and proliferation in human fibroblasts. *J. Cell. Physiol.* 142: 610–614.
31. Marcantonio, E. E., J-L. Guan, J. E. Trevithick, and R. O. Hynes. 1990. Mapping of the functional determinants of the integrin beta 1 cytoplasmic domain by site-directed mutagenesis. *Cell Regul.* 1:597–604.
32. Marguerie, G. A., T. S. Edgington, and E. F. Plow. 1980. Interaction of fibrinogen with its platelet receptor as part of a multistep reaction in ADP-induced platelet aggregation. *J. Biol. Chem.* 255:154–161.
33. Massia, S. P., and J. A. Hubbell. 1991. An RGD spacing of 440 nm is sufficient for integrin $\alpha_v\beta_3$-mediated fibroblast spreading and 140 nm for focal contact and stress fiber formation. *J. Cell Biol.* 114:1089–1100.
34. Munson, P., J., and D. Rodbard. 1980. LIGAND: a versatile computerized approach for characterization of Ligand-binding systems. *Anal. Biochem.* 107:220–239.
35. Nermut, M. V., N. M. Green, P. Eason, S. S. Yamada, and K. M. Yamada. 1988. Electron microscopy and structural model of human fibronectin receptor. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:4093–4099.
36. Niewiarowski, S., E. Regoeczi, and J. F. Mustard. 1972. Adhesion of fibroblasts to polymerizing fibrin and retraction of fibrin induced by fibroblasts. *Proc. Soc. Exp. Biol. Med.* 140:199–204.
37. Nurden, A. T., and J. P. Caen. 1974. An abnormal platelet glycoprotein pattern in three cases of Glanzmann's thrombasthenia. *Br. J. Haematol.* 28:253–260.
38. O'Toole, T. E., J. C. Loftus, E. F. Plow, A Glass, J. R. Harper, and M. H. Ginsberg. 1989. Efficient surface expression of platelet GPIIb-IIIa requires both subunits. *Blood.* 74:14–18.
39. O'Toole, T. E., J. C. Loftus, X. Du, A. A. Glass, Z. M. Ruggeri, S. J. Shattil, E. F. Plow, and M. H. Ginsberg. 1990. Affinity modulation of the $\alpha_{IIb}\beta_3$ integrin (platelet GPIIb-IIIa) is an intrinsic property of the receptor. *Cell Regul.* 1:883–893.
40. O'Toole, T. E., D. Mandelman, J. Forsyth, S. J. Shattil, E. F. Plow, and M. H. Ginsberg. 1991. Modulation of the affinity of integrin $\alpha_{IIb}\beta_3$ (GPIIb-IIIa) by the cytoplasmic domain of α_{IIb}. *Science (Wash. DC).* 254:845–847.
41. Otey, C. A., F. M. Pavalko, and K. Burridge. 1990. An interaction between alpha actinin and the beta 1 integrin subunit in vitro. *J. Cell Biol.* 111:721–279.
42. Pardi, R., L. Inverardi, C. Rugarli, and J. R. Bender. 1992. Antigen-receptor complex stimulation triggers protein kinase C-dependent

CD11a/CD18-cytoskeleton association in T lymphocytes. *J. Cell Biol.* 116:1211–1220.

43. Parise, L. V. 1989. The structure and function of platelet integrins. *Curr. Opin. Cell Biol.* 1:947–952.
44. Phillips, D. R., I. F. Charo, L. V. Parise, and L. A. Fitzgerald. 1988. The platelet membrane glycoprotein IIb-IIIa complex. *Blood.* 71:831–843.
45. Plow, E. F., and M. H. Ginsberg. 1981. Specific and saturable binding of plasma fibronectin to thrombin-stimulated human platelets. *J. Biol. Chem.* 256:9477–9482.
46. Prater, C. A., J. Plotkin, D. Jaye, and W. A. Frazier. 1991. The properdin-like type I repeats of human thrombospondin contain a cell attachment site. *J. Cell Biol.* 112:1031–1040.
47. Reszka, A. A., Y. Hayashi, and A. F. Horwitz. 1992. Identification of amino acid sequences in the integrin $\beta 1$ cytoplasmic domain implicated in cytoskeletal association. *J. Cell Biol.* 117:1321–1330.
48. Ruoslahti, E. 1991. Integrins. *J. Clin. Invest.* 87:1–5.
49. Salomon, D., O. Ayalon, R. Patel-King, R. O. Hynes, and B. Geiger. 1992. Extrajunctional distribution of N-cadherin in cultured human endothelial cells. *J. Cell Sci.* 102:7–17.
50. Santoro, S. A. 1986. Identification of a 160,000 dalton platelet membrane protein that mediates the initial divalent cation-dependent adhesion of platelets to collagen. *Cell.* 46:913–920.
51. Savage, B., and Z. M. Ruggeri. 1991. Selective recognition of adhesive sites in solid phase fibrinogen by GPIIb-IIIa on unstimulated platelets. *J. Biol. Chem.* 266:11226–11233.
52. Schiro, J. A., B. M. C. Chan, W. T. Roswit, P. D. Kassner, A. P. Pentland, M. E. Hemler, A. Z. Eisen, and T. S. Kupper. 1991. Integrin $\alpha^2\beta_1$ (VLA-2) mediates reorganization and contraction of collagen matrices by human cells. *Cell.* 67:403–410.
53. Schwartz, M. A. 1992. Transmembrane signalling by integrins. *Trends Cell Biol.* 2:304–308.
54. Shattil, S. J., J. A. Hoxie, M. Cunningham, and L. F. Brass. 1985. Changes in the platelet membrane glycoprotein IIb-IIIa complex during platelet activation. *J. Biol. Chem.* 260:11107–11114.
55. Shaw, L. M, J. M. Messier, and A. M. Mercurio. 1990. The activation dependent adhesion of macrophages to laminin involves cytoskeletal anchoring and phosphorylation of the alpha 6 beta 1 integrin. *J. Cell Biol.* 110:2167–2174.
56. Solowska, J., J-L. Guan, E. E. Marcantonio, J. E. Trevithick, C. A. Buck, and R. O. Hynes. 1989. Expression of normal and mutant avian integrin subunits in rodent cells. *J. Cell Biol.* 109:853–861.
57. Solowska, J., J. M. Edelman, S. M. Albelda, and C. A. Buck. 1991. Cytoplasmic and transmembrane domains of integrin β_1 and β_3 subunits are functionally interchangeable. *J. Cell Biol.* 114:1079–1088.
58. Takada, Y., J. Ylänne, D. Mandelman, W. Puzon, and M. H. Ginsberg. 1992. A point mutation of integrin β_1 subunit blocks binding of $\alpha_5\beta_1$ to fibronectin and invasin but not recruitment to adhesion plaques. *J. Cell Biol.* 119:913–921.
59. Tamura, R. N., H. M. Cooper, G. Collo, and V. Quaranta. 1991. Cell type-specific integrin variants with alternative alpha chain cytoplasmic domains. *Proc. Natl. Acad. Sci. USA.* 88:10183–10187.
60. Weisel, J. W., C. Nagaswami, G. Vilaire, and J. S. Bennett. 1992. Examination of the platelet membrane glycoprotein IIb-IIIa complex and its interaction with fibrinogen and other ligands by electron microscopy. *J. Biol. Chem.* 267:16,637–16,643.
61. Weiss, H. J., V. T. Turitto, and H. R. Baumgartner. 1986. Platelet adhesion and thrombus formation on subendothelium in platelets deficient in glycoproteins IIb-IIIa, Ib, and storage granules. *Blood.* 67:322–330.
62. Wencel-Drake, J. D., E. F. Plow, T. J. Kunicki, V. L. Woods, D. M. Keller, and M. H. Ginsberg. 1986. Localization of internal pools of membrane glycoproteins involved in platelet adhesive responses. *Am. J. Pathol.* 124:324–334.
63. Woods, V. L., E. H. Oh, D. Mason, and R. McMillan. 1984. Autoantibodies against the platelets glycoprotein IIb/IIIa complex in patients with chronic ITP. *Blood.* 63:368–375.
64. Ylänne, J., M. Hormia, M. Jarvinen, T. Vartio, and I. Virtanen. 1988. Platelet glycoprotein IIb/IIIa complex in cultured cells. Localization in focal adhesion sites in spreading HEL cells. *Blood.* 72:1478–1486.
65. Ylänne, J., D. A. Cheresh, and I. Virtanen. 1990. Localization of β_1, β_3, α_5, α_v, and α_{IIb} subunits of the integrin family in spreading human erythroleukemia cells. *Blood.* 76:570–577.